

Scotland's Rural College

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Published in:
Poultry Science

DOI:
[10.3382/ps/pex413](https://doi.org/10.3382/ps/pex413)

First published: 01/04/2018

Document Version
Peer reviewed version

[Link to publication](#)

Citation for pulished version (APA):

Khattak, FM., Paschalis, V., Green, M., Houdijk, JGM., Soultanas, P., & Mahdavi, J. (2018). TYPELEX® Chelate, a novel feed additive, inhibits *Campylobacter jejuni* biofilm formation and cecal colonization in broiler chickens. *Poultry Science*, 97(4), 1391 - 1399. <https://doi.org/10.3382/ps/pex413>

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3 **TYPLEX[®] Chelate inhibits *Campylobacter Jejuni* Biofilm Formation and Caecal**
4 **Colonization in Broiler Chickens**

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12 *Scientific section for paper: Microbiology and Food Safety*
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INHIBITION OF *CAMPYLOBACTER* BIOFILM AND COLONISATION

TYPLEX[®] Chelate inhibits *Campylobacter Jejuni* Biofilm Formation and Caecal Colonization in Broiler Chickens

ABSTRACT

Reducing *Campylobacter* spp. carriage in poultry is challenging, but essential to control this major cause of human bacterial gastroenteritis worldwide. Although much is known about the mechanisms and route of *Campylobacter* spp. colonization in poultry the literature is scarce on antibiotic-free solutions to combat *Campylobacter* spp. colonization in poultry. *In vitro* and *in vivo* studies were conducted to investigate the role of TYPLEX[®] Chelate (ferric tyrosine), a novel feed additive, in inhibiting *Campylobacter jejuni* (*C. jejuni*) biofilm formation and reducing *C. jejuni* and *Escherichia coli* (*E. coli*) colonization in broiler chickens at market age. In an *in vitro* study, the inhibitory effect on *C. jejuni* biofilm formation using a plastic bead assay was investigated. The results demonstrated that TYPLEX[®] Chelate significantly reduces biofilm formation. In an *in vivo* study, 800 broilers (one-day old) were randomly allocated to 4 dietary treatments in a randomised block design, each having 10 replicate pens with 20 birds per pen. At day 21, all birds were challenged with *C. jejuni* via seeded litter. At day 42, caecal samples were collected and tested for volatile fatty acid (VFA) concentrations, *C. jejuni* and *E. coli* counts. The results showed that TYPLEX[®] Chelate reduced the carriage of *C. jejuni* and *E. coli* in poultry by 2 and 1 log₁₀ per gram caecal sample, respectively, and increased caecal VFA concentrations. These findings support TYPLEX[®] Chelate as a novel non-antibiotic

feed additive that may help produce poultry with a lower public health risk of *Campylobacteriosis*.

KEY WORDS

Campylobacter, biofilm, volatile fatty acid, feed additive, food safety

INTRODUCTION

Campylobacter spp. infections are a major cause of human bacterial gastroenteritis and pose a serious health burden worldwide, accounting for 400-500 million cases of diarrhoea each year (Ruiz-Palacios, 2007). A significant proportion of health care costs are associated with sequelae linked to *Campylobacteriosis* such as Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome (WHO, 2013). Despite several government programs and awareness campaigns to reduce *Campylobacter* spp. little reduction is reported in the numbers of bacterium in animals and/or animal products in retail outlets (Robyn et al., 2015). It is estimated that foodborne transmission contributes to 58% of the global disease burden (Hald et al., 2015).

Chicken, pork and beef are reported as significant foodborne sources of *Campylobacter* with presence of the pathogen at high concentrations found throughout the food chain (Miller and Mandrell, 2005). The strong adhesion capability of *Campylobacter* strains could partly explain the rapid cross-contamination or re-contamination of food products, and may be the most significant mode of survival for *Campylobacter* in the food chain (Sulaeman et al., 2010).

Campylobacter infection is dependent on motility mediated by polar flagella A (**FlaA**) and adhesion and biofilm promoting ability of the Major Outer Membrane Protein (**MOMP**) (Ashgar et al., 2007; Muller et al., 2007 and Min et al., 2009; Mahdavi et al., 2014). Considering such idiosyncrasy, it is easy for *Campylobacter* to

70 colonize live animals and survive transitionally in the food chain or in biofilms before
71 reaching the intestinal tract of humans (Buswell et al., 1998; Trachoo and Frank,
72 2002; Miller and Mandrell, 2005; Lehtola et al., 2006; Sanders et al., 2008).

73 Biofilms are accumulations of microorganisms embedded in an extracellular matrix
74 (**ECM**) which adheres to solid biological or non-biotic surfaces (Costerton, 1995;
75 Kalmokoff et al., 2006). The ECM comprises proteins, polysaccharides, nucleic acids
76 and phospholipids (Stoodley et al., 2004). Foodborne bacterial pathogens can either
77 form biofilms on inert surfaces or can reach and integrate into pre-established
78 biofilms.

79 Increase in caecal volatile fatty acid (VFA) concentrations, indicating fermentation by
80 anaerobic bacteria, are found to be negatively correlated with the number of
81 Enterobacteriaceae in broiler chickens (Van Der et al., 2000; Kubena et al., 2001).

82 Several strategies such as reduction of environmental exposure to *Campylobacter* by
83 hygiene and biosecurity measures, water treatment, use of plant-derived feed
84 additives, use of bacteriophage, bacteriocin therapies, vaccination, passive
85 immunization, use of pre- and probiotics and genetic selection have been investigated
86 to control on-farm *Campylobacter* contamination (Sahin et al., 2003; Chaveerach et
87 al., 2004; Carrillo et al., 2005; Calderon-Gomez et al., 2009; Lin, 2009; Buckley et
88 al., 2010; Svetoch and Stern, 2010; Hermans et al., 2010; Hermans et al., 2011).

89 Despite all these efforts *Campylobacter* remains a major cause of human
90 gastroenteritis and a priority for the development of new control strategies. In
91 addition, use of antibiotics in both animal and human medicine can influence the
92 development of antibiotic-resistant *Campylobacter* and is increasingly becoming a
93 challenge for food safety and public health (Luangtongkum et al., 2009). The authors
94 postulated that the novel feed additive, TYPLEX[®] Chelate, a synthetic complex of L-

tyrosine and Fe (III), exerts a unique action on enteropathogens by preventing the formation of biofilms at chyme/mucosal and other interfaces. Based on the non-antimicrobial nature of ferric tyrosine the current studies were designed to demonstrate that TYPLEX[®] Chelate inhibits *C. jejuni* biofilm formation (*in vitro*), increases caecal VFA concentrations (*in vivo*) and thus has an ability to reduce caecal colonization of *C. jejuni* and *E. coli* in broilers at slaughter.

MATERIALS AND METHODS

Feed additive

TYPLEX[®] Chelate (Akeso Biomedical, Inc., Waltham, MA), a complex containing tyrosine and iron (ferric tyrosine), was the novel feed additive used in these studies.

Experimental diets

Basal iso-nitrogenous and iso-energetic wheat-soyabean meal control diets (T₁) were manufactured as one batch for each feeding phase *i.e.* starter (day 0-21) and grower (day 21-42). Three additional treatments (T₂ to T₄) were generated by addition of TYPLEX[®] Chelate to T₁ at 0.02, 0.05 and 0.20 g/kg feed, respectively. Diets were manufactured with coccidiostats but contained no veterinary antibiotics. The ingredients, premixes and the calculated analyses of the starter and grower diets are presented in Table 1. Diets were analysed for dry matter, nitrogen, ether extract and iron. Total nitrogen content of diet was determined by the combustion method (AOAC Method 968.06) whereas ether extract was determined in a soxhlet extractor (AOAC Method 922.06). Iron content was determined using Inductively Coupled Plasma – Optical Emission Spectroscopy (AOAC Method 990.08) following digestion, in turn, in concentrated Nitric and Hydrochloric acid. Coloured tracers (Micro-Tracers Inc, San Francisco) were added to TYPLEXTM chelate at 10% w/w, to

enable visual confirmation of TYPLEXTM chelate content and uniform mixing in feed samples.

In vitro assessment of C. jejuni biofilm inhibition in a simulated gut environment using a plastic bead assay

A plastic bead assay (O'Toole and Kolter, 1998; Stepanovic et al., 2000) with some modifications for suitability with *C. jejuni* was carried out *in vitro* to show that the TYPLEX[®] Chelate extracted from the experimental diets has an inhibitory effect on *C. jejuni* biofilm formation.

Extraction of TYPLEX[®] chelate from the experimental diets. 10 g aliquots of feed from all experimental diets (T₁ to T₄) for each phase were homogenised to very fine particles (around 100 µm) and mixed with 50 ml (1:5) of buffer (20 mM KCl/HCl; pH 3.4). The feed/buffer mixtures were autoclaved (high-pressure saturated steam at 121°C for around 15–20 minutes, 3 cycles). The high heat and pressure of the autoclave resulted in extraction of the TYPLEX[®] Chelate from the feeds while retaining its physical properties. The resulting suspensions were then filtered using filter paper (25 µm filter). The pH of all filtered samples was measured and found to be between 6.2 to 6.5.

Bead Assay. *C. jejuni* strain NCTC11168 was grown overnight in 3 ml of sterile brain heart infusion (BHI) broth under microaerophilic conditions (85% nitrogen, 10% carbon dioxide and 5% oxygen) at 42°C on modified *Campylobacter*-selective charcoal cefoperazone desoxycholate (CCDA). The optical density at a wavelength of 600 nm (OD₆₀₀) of these cultures were measured, then they were used to inoculate a feed extract + BHI suspension (1:4 dilution) to achieve a final OD₆₀₀ of 0.02.

Two sterile plastic beads per dietary treatment were placed into 2.5 ml of BHI, with 0.5 ml of the extracted feeds and inoculated with *C. jejuni*. The beads were then incubated at 42°C for 48 hr. Following incubation, the beads were gently washed twice (5x dipping each) in phosphate buffered saline (**PBS**), placed into 1 ml of PBS and vigorously vortexed for 30 s prior to centrifuging at 3000 rpm. The PBS containing bacterial cells released from the biofilm was serially diluted and 3 x 5 µl aliquots of each dilution were spotted onto **CCDA** agar plates for quantification (Thermo-Fisher plates, 3 plates/bead, two beads/treatment, 6 samples/treatment). The plates were subsequently incubated microaerophilically at 42°C for 48 hrs and colonies were counted and expressed as cfu/ml. The same experiment was carried out in triplicate.

Seedler litter challenge to colonise chicken with C. jejuni in vivo

To confirm *in vitro* findings, an *in vivo* study was designed to investigate the efficacy of TYPLEX[®] Chelate under farm conditions. A total of 800 male broiler chickens (Ross 308) were allocated randomly to pens in 4 dietary treatments (T₁ to T₄), with pens distributed using a randomized complete block design. Each treatment had 10 replicate floor pens with 20 chicks per pen. Birds were reared on fresh wood shavings in clean pens having European Union maximum stocking density at 42 d of 38 kg/m². At 21 days of age all birds were challenged with *C. jejuni* seeded litter. Diets were fed *ad libitum* for 42 days in mash form; body weight and feed intake of each replicate pen were recorded to calculate the global zootechnical data (days 0-39). At the end of the study (day 42), all birds were humanely killed through cervical dislocation and caecal samples were collected from 10 birds/pen and used for *C. jejuni* and *E. coli* enumeration, and 2 birds/pen for caecal VFA analysis.

The *in vivo* study (AU AE 37-2016) was carried out under the Animals Scientific Procedures Act (1986) and approved by the ethical review committee of Scotland's Rural College (SRUC).

Seeded litter challenge procedure. Approximately 20 kg of used poultry litter was taken from a recently completed broiler study. The litter (not tested for any pathogen) was placed in an oven at 80°C until a constant weight was obtained, then divided into 400 g batches in forty trays (each tray was approximately 38 × 28 × 8 cm). Each dried reused litter tray was reconstituted with 1000 ml of deionised water. Trays were then seeded with a mixture of Mueller-Hinton (MH) broth, *Campylobacter* suspension (4.5×10^5 cfu *C. jejuni*/ml) and dried hen droppings (20 ml: 10 ml: 10g). The hen droppings were dried in the same way as the litter. *Campylobacter* isolates were obtained from caecal samples taken from three different commercial poultry farms and stored at -80°C in bead cryopreservation vials (Technical Service Consultants, UK). *C. jejuni* (individual strains were not identified) were resuscitated on Blood Agar No. 2 with Horse Blood (BA) plates (Oxoid, UK). These cultures were used to prepare lawn plates on further BA plates, incubated for 40 – 48 hrs at 41.5°C, in boxes with a microaerophilic atmosphere generation system (CampyGen, Oxoid, UK). The lawn plates were harvested by adding 5 ml MH broth, gently detaching the culture with a sterile spreader and decanting to a container. The suspension was then adjusted with further MH broth to OD₆₀₀ of 0.19 – 0.21 (approximately 1.5×10^5 cfu.ml⁻¹). The bacterial suspension, broth and droppings were mixed and spread evenly on the top of the litter tray. The tray was then placed in a pen near the feeder.

Isolation and enumeration of *C. jejuni* from caeca. A sterile scalpel was used to cut off the blind end of both caecal sacks from each chicken. For each sample, 0.5g

of content from each caecal sac (in total 1g) was weighed out into sterile Universal bottles. At each sampling, a total of 2g caecal content was diluted with 4 ml of sterile Maximum Recovery Diluent (**MRD**), which was added to each Universal container and mixed thoroughly. This constituted the 1:2 dilutions (w/v). A further 8 serial dilutions were made in MRD. Then, 5-10 μ L of each dilution was spotted on CCDA, MRS, Brilliance or chromogenic plates (Oxoid PO0119) and left to dry. Plates were incubated microaerophilically at $41.5^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$ for 24-48 hrs. Following incubation, plates were assessed for the presence or absence of thermotolerant *Campylobacter* species. In addition, plates of an appropriate dilution level were selected and colonies enumerated. As a confirmatory measurement, two colonies from each presumptively positive plate were selected and sub-cultured onto paired blood agar plates (Oxoid PB0114). These plates were incubated at 37°C for 48 hrs, one plate aerobically and one plate microaerophilically. The presence of *C. jejuni* was indicated by lack of growth aerobically and colonies with *Campylobacter* morphology that grew microaerophilically. In addition, Gram stains were performed on all presumptively *Campylobacter* positive samples. Oxidase strips (Oxoid MB0266) were used to further confirm that the samples were oxidase positive.

In case of pre-inoculation testing, at day 16 cloacal swab (1 bird/pen) and overshoes (1 overshoe/pen) were tested for presence and absence of *C. jejuni* using the same procedure as reported for *C. jejuni* enumeration.

Caecal volatile fatty acid analysis. The caecal digesta were gently flushed out in 30 ml universal sample containers and immediately stored at -80°C until analysed. Samples were ground in the presence of solid CO_2 to ensure homogeneity. They were then shaken with a known volume of water to extract the fatty acids. The extracts were spiked with 4-methylvaleric acid as an internal standard and then passed to the

Gas Chromatography (HP 5890 Series II GC; Agilent J & W 30m × 0.535mm × 1.00 micon HP-FFAP column; FID detector) to determine the individual component composition by comparison with a series of standard solutions which were also spiked with an internal standard. Acetic, *n*-butyric, propanoic, *n*-valeric, *iso*-butyric and *iso*-valeric acid were detected and results were expressed as mg/kg.

Statistics

For the *in vitro* tests, a single 5 µl aliquot extracted from a bead in a serially-diluted manner, grown on a CDDA plate was the statistical unit of measurement. A randomized complete block design with ten blocks and four treatments was used for *in vivo* studies. The individual bird sampled was the experimental unit for microbiological (*C. jejuni* and *E. coli*) and VFA analysis whereas the pen was the experimental unit for growth performance data. The microbiological data were log₁₀ transformed prior to analysis. Data obtained were subjected to Analysis of Variance (ANOVA) using a GenStat 16 statistical software package (IACR, Rothamstead, Hertfordshire, UK). Significance between treatments was determined using orthogonal polynomial contrasts. Where correlations are presented (identified using Genstat), all r^2 are significant to at least $P < 0.05$ unless otherwise stated. All statements of significance are based on the probability level of $P \leq 0.05$.

RESULTS

Nutrient composition and analysed TYPLEX[®] Chelate of the experimental diets were within the expected range and are presented in Tables 1 and 2 respectively.

Inhibitory impact of extracted TYPLEX[®] Chelate from feeds on C. jejuni biofilm formation

In vitro data showed that the addition of TYPLEX[®] Chelate significantly reduced the ability of *C. jejuni* to adhere to plastic beads and form a biofilm (Table 3). For the

242 starter diet, the addition of TYPLEX[®] Chelate extract at all concentrations reduced
243 linearly the number of *C. jejuni* cells compared to the control group (0.5, 0.3 and 0.5
244 log₁₀ cfu/g reduction, respectively, $P < 0.001$). Similar results were observed for the
245 grower diet; the addition of TYPLEX[®] Chelate extract at 0.02 g/kg, 0.05 g/kg and 0.2
246 g/kg reduced linearly the number of *C. jejuni* cells compared to the control group (0.4,
247 1.4 and 1.0 log₁₀ cfu/g reductions, respectively, $P < 0.001$).

248 ***Campylobacter infection and bird performance***

249 The seeded litter challenge model was successful in horizontal transfer of *C. jejuni* in
250 birds within pens. Pre-inoculation cloacal swabs and samples cultured from overshoes
251 were negative, whereas all caecal samples from all pens cultured positive for *C.*
252 *jejuni*. The growth performance of birds during the starter (days 0-21), grower (days
253 21 to 39) and overall growth period (days 0 to 39) is presented in Table 4. During the
254 pre-challenge (starter phase), average weight gain (AWG) of all birds fed the
255 TYPLEX[®] Chelate supplemented diet were consistently higher compared to the
256 T1control group ($P < 0.05$). However, the differences were only significant when
257 birds fed T2 and T4 (0.02 and 0.20g/kg TYPLEX[®] Chelate) were compared with T1
258 ($P < 0.05$). This improvement in AWG also resulted into 3.6% lower ($P < 0.05$)
259 mortality adjusted feed conversion ratio (FCR) values compared with T1. During the
260 *Campylobacter* challenged period (grower phase) all birds fed TYPLEX[®] Chelate
261 (T2, T3 and T4) had 19.6% higher ($P < 0.05$) AWG compared to T1 (control group).
262 This increase in AWG was also translated into 5.7% improvement in FCR of all birds
263 fed TYPLEX[®] Chelate (T2, T3 and T4) supplemented diets compared to control
264 group (T1). The average feed intake (AFI) over the entire trial period was lower ($P <$
265 0.05) for T3 (0.05g/kg TYPLEX[®] Chelate) compared to T1 control. This shift in AFI
266 resulted in significantly lower ($P < 0.05$) FCR for birds in T3 group compared to

control. The overall growth performance depicted similar trends and showed 2.9 and 4.9% linear improvement ($P < 0.05$) in AWG in birds fed T2 and T3 (0.02 and 0.20g/kg TYPLEX[®] Chelate), respectively and 4.1 % lower ($P < 0.05$) FCR for birds fed all TYPLEX[®] Chelate diets (T2, T3 and T4) compared to control (T1). The overall AWG and FCR values were positively correlated ($r^2 = 0.693, 0.822$ respectively; $P < 0.05$) whereas, AFI showed no correlation ($r^2 = 0.03$; $P > 0.05$) with the inclusion level of TYPLEX[®] Chelate in the diet. The birds remained healthy through out the experimental period, and the percentage mortality for treatment 1 to 4 was 6.0, 6.5, 3.5 and 6.5% respectively. Mortality was not associated with treatment ($P > 0.05$).

Effect of TYPLEX[®] Chelate on caecal C. jejuni and E. coli colonization.

Microbiological analyses of caecal samples showed a 0.8, 2.1 and 2.1 log₁₀ cfu/g linear reduction ($P < 0.05$) in *C. jejuni* colonization in TYPLEX[®] Chelate supplemented T2, T3 and T4 treatment groups respectively compared to T1 control (Table 5). Similarly, 0.6, 0.8 and 1.2 log₁₀ cfu/g linear reduction ($P < 0.05$) in *E. coli* counts was recorded in birds that received 0.02, 0.05 and 0.20 g/kg TYPLEX[®] Chelate (T2, T3 and T₄) compared with T1 control (Table 5). The *Campylobacter* spp. and *E. coli* counts were positively correlated with the inclusion level of TYPLEX[®] Chelate ($r^2 = 0.931, 0.952$, respectively; $P < 0.05$).

Effect of dietary TYPLEX[®] Chelate on caecal VFA concentrations.

Individual and total VFA concentrations in the caecal digesta are presented in Table 6. Total VFA concentrations increased by 31 and 26% ($P < 0.05$) in birds fed 0.02 and 0.05 g TYPLEX[®] Chelate /kg feed, respectively, compared to the control group. This increase was due to a general increase in most acids. Thus, feeding birds diets containing 0.02 and 0.05 g TYPLEX[®] Chelate/kg feed increased ($P < 0.05$) the

concentration of acetic acid by 26 and 24 %, respectively, compared to the non-supplemented T1 control. No additional increase in acetic acid concentration was observed when the inclusion rate of TYPLEX[®] Chelate was further increased from 0.05 to 0.20 g/kg feed. Propionic acid concentrations were also consistently higher in birds fed TYPLEX[®] Chelate diets, but the differences were not significant ($P > 0.05$) when compared with the T1 control. Differences in the concentration of n-butyric acid were more profound and the concentration linearly increased ($P < 0.05$) by 75, 66 and 41% in birds T2, T3 and T4 fed diets containing 0.02 and 0.05 and 0.20g TYPLEX[®] Chelate /kg feed, respectively, compared with T1 control . Also, n-valeric, *iso*-butyric and *iso*-valeric acids were produced in very small quantities, but followed the same trend, with significantly greater concentrations in birds fed T3 diets containing 0.05g TYPLEX[®] Chelate/kg feed compared to T1 control.

DISCUSSION

The public health significance of *C. jejuni* infection and emergence of multi-antibiotic resistant species of *Campylobacter* demands development of new control strategies in addition to farm biosecurity measures to lower carriage of *C. jejuni* in live animals. To our knowledge, this is the first study to report that TYPLEX[®] Chelate inhibits *C. jejuni* biofilm formation. The *in vitro* assay showed that TYPLEX[®] Chelate (extracted from experimental diets) in a simulated gut environment reduced the ability of *C. jejuni* to adhere to plastic beads and form a biofilm.

It is known that MOMP binds to multiple host cell membranes by promoting biofilm formation and auto-aggregation. It is the biofilm-forming ability of *C. jejuni* that enables the organism to survive in the environment and enter the food chain (Joshua et al., 2006). Based on our *in vitro* results it is likely that TYPLEX[®] Chelate inhibits FlaA and MOMP-mediated adhesion of *C. jejuni* as evidenced by the reduction in

biofilm formation. The ability of *C. jejuni* to attach to surfaces and grow in biofilms, where they are protected from antibiotics, biocides, and other chemical or physical challenges, is a key factor in persistence of infection in humans (Costerton et al., 1999; Stewart, 2002; Stoodley et al., 2004). As the TYPLEX® Chelate inhibits biofilm formation it can therefore be helpful in farming and food processing to reduce cross-contamination of food products. This strategy offers an antibiotic-free method which will not encourage the emergence of resistance in pathogenic bacteria within the host organism. The inhibitory effect of TYPLEX® Chelate on biofilm formation showed the same pattern in starter and grower diets.

The birds in the current study were challenged at day 21 because *Campylobacter* spp. is rarely detected in commercial flocks of less than 3 weeks of age, regardless of production methods (Kazwala et al., 1990), species (Allen et al., 2011; Umar et al., 2016) and biosecurity measures (Allain et al., 2014). The lag phase in colonization of poultry, even in the presence of positive birds suggests that a biological mechanism of colonisation resistance may be present in young birds due to maternal antibodies (Cawthraw and Newell, 2010). The relatively dry litter in the poultry house may also limit the ability of *Campylobacter* to survive in the small volume of excreta produced by birds in the first few weeks of life (Sparks, 2016). However, when the flock is infected, the majority of birds become colonized within 4 to 7 days after infection of the first bird (Sahin et al., 2015), and the overall prevalence rises as high as 100% at slaughter age (Barrios et al., 2006), without any apparent clinical manifestations in the chicken host (Kaino et al., 1988).

The incidence of human campylobacteriosis is often associated with consumption of poultry products contaminated with *C. jejuni* and this, in turn, is linked to the number of *C. jejuni* present in the caeca of the bird (Wagenaar et al, 2006; Neal-McKinney et

al., 2014). As few as 500 cells of *C. jejuni* can cause infection (Black et al., 1988). There is no evidence for a “safe” level of *Campylobacter* contamination, as the minimum infectious dose will always be strain-specific. In general, it is considered that the risk of *Campylobacteriosis* increases as the number of *Campylobacter* on the bird increases (EFSA, 2009). Therefore, it is possible to reduce the incidence of human infection by lowering the number of *C. jejuni* in birds bound for the food supply. In this study, use of TYPLEX[®] Chelate in broiler diets at 0.05 and 0.20 g/kg feed reduced the counts of caecal *C. jejuni* by up to 2 log₁₀ cfu/g sample the counts of caecal *C. jejuni* in broilers aged 42 days. According to food safety risk analysis studies, this level of reduction in colonization may be able to reduce the public health risk associated with human campylobacteriosis (Rosenquist et al., 2003; EFSA, 2011). One of the mechanisms known to reduce *Enterobacteriaceae* (Gram-negative bacteria) in the intestinal microflora is the bacteriostatic effect of VFA’s in the caeca (Van Der et al., 2000). The current study showed that feeding broilers with diets supplemented with 0.02 and 0.05g TYPLEX[®] chelate/kg feed resulted in increased ($P < 0.05$) caecal concentrations of total VFA, mainly due to higher production of acetic and *n*-butyric acid. High concentrations of VFA are indicative of fermentations by obligate anaerobic bacteria, reported to be an important source of energy for enterocytes and vital for intestinal health (Sunkara et al., 2012). It has been reported that increased concentrations of VFAs lower the intestinal pH, which is associated with suppression of pathogens (Kubena et al., 2001, Rehman et al., 2007). VFAs not only affect host functions but also serve as a carbon source for the endogenous bacteria and at high concentrations can exhibit toxic effects on bacteria. Chickens are omnivores, and the diversity of microbial communities in their intestinal tracts is related to their life-style. It has been proposed that the different nutritional

367 requirements for maintaining homeostasis of the microbiota communities in the gut
 368 would be due to different fermentable substrates available in the terminal ilea (Fang et
 369 al., 2012). Among the bacterial fermentation end-products in the chicken caecum,
 370 butyrate is of particular importance because of its nutritional properties for epithelial
 371 cells and pathogen inhibitory effects in the gut (Sun and O’Riordan, 2013). In the
 372 current study, the reduction in the proportion of acetic, butyric and valeric acids in the
 373 challenged unsupplemented control group (T1) could be due to *C. jejuni*. It has been
 374 reported that *Campylobacter* colonization reduces butyrate, iso-butyrate, valerate, and
 375 iso-valerate in the caecum (Awad et al., 2016).

376 Furthermore, translocation of *E. coli* to the liver, spleen and caecum increases in birds
 377 infected with *C. jejuni* (Awad et al., 2016). Results from some of the epidemiological
 378 studies also reported an increase of *E. coli* in chicken carcasses that were infected
 379 with *Campylobacter* (Duffy et al., 2014). The results from this study suggest that
 380 *Campylobacter* infection may have an influence on the development of other
 381 microbial populations, such as *E. coli*, illustrated by the data on TYPLEX[®] Chelate,
 382 used at higher doses. At 0.20 g/kg feed, TYPLEX[®] Chelate not only caused a 2.1
 383 log₁₀ cfu/g reduction in caecal *C. jejuni* colonization but also resulted in 1 2 log₁₀
 384 cfu/g reduction in *E. coli* populations. In a previous study, we found that inclusion of
 385 TYPLEX[®] Chelate in broiler diets without coccidiostats caused a significant
 386 reduction in coccidial oocyst counts and elevated the microbial dominance at 52-54%
 387 Guanine + Cytosine in comparison with non-supplemented diets, indicative of high-
 388 performing healthy birds (Khattak et al., 1997). These results support the notion that
 389 TYPLEX[®] Chelate may reduce the activities of pathogenic bacteria but favour
 390 beneficial bacteria and thus enhance growth performance. In addition, the assessment
 391 of minimum inhibitory concentrations (**MIC**) of TYPLEX[®] Chelate to *C. jejuni*, *E.*

392 *coli*, and *Salmonella enterica* showed that TYPLEX[®] Chelate did not inhibit the
393 growth of these pathogens (personal communications with Dr Juha Apajalahti;
394 Alimetrix Group Ltd). The MIC values were higher than 400mg/l for *C. jejuni* and
395 higher than 200mg/l for *E. coli* and *Salmonella*, indicating that none of the enteric
396 pathogens tested were inhibited by TYPLEX[®] Chelate at concentrations relevant for
397 animal feed applications. It is possible that the TYPLEX[®] Chelate does not kill these
398 pathogens but reduces their ability to adhere to the gut mucosa and thus lower their
399 chances to compete with beneficial bacteria.

400 The addition of TYPLEX[®] Chelate to the diet consistently improved broiler AWG
401 and FCR compared to non-supplemented control birds. It has been reported that *C.*
402 *jejuni* can cause a significant decrease in weight gain of poultry (Awad et al., 2014a)
403 and that there is a highly significant association between *Campylobacter* positivity
404 and poorer FCR (Sparks, 2016). *Campylobacter* infection is found to significantly
405 downregulate the gene expression of the sodium/glucose cotransporter (SGLT-1),
406 peptide transporter (PepT-1), glucose transporter (GLUT-2), cationic amino acid
407 transporter (CAT-2), excitatory amino acid transporter (EAAT-3) and the L-type
408 amino acid transporter (y⁺LAT-2) in different parts of the gut (Awad et al., 2014b).
409 Such decreased nutrient absorption not only explains the negative effect of
410 *Campylobacter* colonization on body weight but this could be crucial for the
411 persistence of *Campylobacter* itself. To colonize and invade, *C. jejuni* bacteria require
412 adequate nutrients, mainly amino acids and Ca²⁺ (Fang et al., 2012; Awad et al.,
413 2015). Therefore, reduction in the intestinal uptake of nutrients by an avian host may
414 increase carbon and nitrogen sources that are essential for bacterial growth (Guccione
415 et al., 2008). The present study suggests that TYPLEX[®] Chelate may also promote
416 growth performance by enhancing intestinal nutrient absorption and reducing

colonization of *C. jejuni*. However, in addition to on-farm environmental conditions and host immune response (Lin, 2009), any negative effects on broiler body weights due to *Campylobacter* could also be strain-specific as different isolates of *C. jejuni* can have different colonization potential (Hermans et al., 2011).

In conclusion, the data from the current studies suggest that TYPLEX® Chelate reduces gut colonization of *C. jejuni* by inhibiting biofilm formation, increases the bacteriostatic effect of VFA and improve intestinal absorptive function. By reducing their adhesion power, it is likely that the spread of *C. jejuni* through other biological and non-biological routes would be reduced. In addition, disabling biofilm resistance may enhance the ability of existing antibiotics to fight infections that are refractory to current treatments and eventually help to reduce human cases of *Campylobacteriosis*.

ACKNOWLEDGEMENT

The work was funded by Akeso Biomedical, Inc., Waltham, MA, USA and the Scottish government (RESAS).

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636

637 **Table 1.** Composition of basal diets

Ingredients (% unless otherwise stated)	Starter diets (d 0-21)	Grower diets (d 21-42)
Barley	10.415	8.315
Wheat	50	55
Soya Ext Hipro	26	23
Full fat Soya Cherwell	5	5
L Lysine HCl	0.4	0.3
DL-methionine	0.4	0.35
L-threonine	0.15	0.15
Soya Oil	4	4.5
Limestone Trucal 52	1.25	1.25
Monocalcium phosphate	1.5	1.25
Salt	0.25	0.25
Sodium bicarbonate	0.15	0.15
Broiler Premix*	0.4	0.4
Robenz 66G Premix (robenidine coccidiostat)	0.05	0.05
Ronozyme WX (polysaccharidase enzymes)	0.02	0.02
Ronozyme P 5000 (CT) (phytase enzymes)	0.015	0.015
Total	100%	100%
Calculated analysis		
Fat (ether extract)	6.39	6.85
Protein	21.84	20.64
Fibre	3.08	3.02
Ash	6.02	5.68
ME-P	12.73	13.04
Total lysine	1.43	1.28
Available lysine	1.33	1.19
Methionine	0.69	0.62
Total methionine and cysteine	1.03	0.95
Threonine	0.91	0.86
Tryptophan	0.25	0.23
Calcium	0.95	0.91
Phosphorus	0.73	0.66
Available phosphorus	0.48	0.42
Sodium chloride	0.30	0.30

Sodium	0.17	0.17
Analysed Nutrient composition		
Dry Matter	88.1	88.2
Crude protein†	21.8	20.7
Ether extract	6.66	7.12
Iron (mg/kg)	135	99

*Premix provided per kg: vitamin A, 2400 IU; vitamin D3, 1000 IU; vitamin E, 10,000 IU; vitamin K3, 600 mg/kg; vitamin B1, 400 mg/kg; vitamin B2, 1,400 mg/kg; pantothenic acid, 3,000 mg/kg; nicotinic acid, 10,000 mg/kg; vitamin B6, 1,000 mg/kg; vitamin B12, 3,000 ug/kg; folic acid: 200 mg/kg; biotin: 40 mg/kg; copper, 2,000 mg/kg; zinc, 16,000 mg/kg; manganese, 20,000 mg/kg; iodine, 200 mg/kg; selenium, 40 mg/kg; choline choride, 500 g: No added iron in premix.

† Crude protein = Nitrogen x 6.25.

645 **Table 2.** Calculated and analysed value of TYPLEX[®] Chelate in experimental diets.

Treatment	Calculated Value	Analysed Value of TYPLEX [®] Chelate in starter diets (g/kg)	Analysed Value of TYPLEX [®] Chelate in grower diets (g/kg)
T ₁	0	0	0
T ₂	0.02	0.02	0.02
T ₃	0.05	0.06	0.04
T ₄	0.20	0.19	0.26

646

Table 3. Inhibitory effect of TYPLEX[®] Chelate on *C. jejuni* (NCTC11168) biofilm formation using plastic beads.

Treatment	Starter diet Log ₁₀ cfu/ml	Grower diet Log ₁₀ cfu/ml
T1	5.608	5.573
T2	5.082	5.194
T3	5.285	4.129
T4	5.123	4.583
SEM	0.040	0.076
P-value's for contrast		
T1 versus T2	<0.001	<0.001
T1 versus T3	<0.001	<0.001
T1 versus T4	<0.001	<0.001
Linear	<0.001	

N°. replicates/treatment = 18; cfu = colony forming units.

SEM = standard error of the mean.

T1= Control ((HCl/KCl) ; T2 = 0.02 g/kg TYPLEX[®] Chelate ;T3 = 0.05 g/kg TYPLEX[®] Chelate; T4 = 0.20 g/kg TYPLEX[®] Chelate.

654 Table 4. Effect of dietary treatments on growth performance of broilers¹

655

Item	Starter phase (d 0-21)			Grower phase (d 21-39)			Overall Performance (d0-39)		
	AWG ² (kg/bird)	AFI ³ (kg/bird)	FCR ⁴ (kg/kg)	AWG (kg/bird)	AFI (kg/bird)	FCR (kg/kg)	AWG (kg/bird)	AFI (kg/bird)	FCR (kg/kg)
Treatment									
T1	0.773	1.077	1.400	0.381	2.948	1.611	2.618	4.025	1.544
T2	0.821	1.076	1.314	0.428	2.915	1.579	2.695	3.991	1.495
T3	0.776	1.024	1.327	0.461	2.865	1.533	2.660	3.890	1.471
T4	0.829	1.085	1.316	0.479	2.943	1.543	2.748	4.028	1.472
SEM	0.014	0.016	0.027	0.020	0.036	0.023	0.029	0.047	0.016
P-values for contrast ⁵									
T1 versus T2	0.002	0.979	0.004	0.027	0.361	0.182	0.02	0.484	0.007
T1 versus T3	0.842	0.003	0.012	<0.001	0.031	0.002	0.181	0.009	<0.001
T1 versus T4	<0.001	0.584	0.005	<0.001	0.889	0.007	<0.001	0.94	<0.001
Linear	0.011	0.614	0.010	0.579	0.579	0.002	0.001	0.559	<0.001

656 ¹All means are average of 10 pens per treatment.

657 ² AWG = Average weight gain.

658 ³ AFI = Average feed intake.

659 ⁴ FCR = Feed conversion ratio.

660 T1= Control; T2 = 0.02 g/kg TYPLEX[®] Chelate; T3 = 0.05 g/kg TYPLEX[®] Chelate; T4 = 0.20 g/kg TYPLEX[®] Chelate.

661 ⁵Significance level ($P \leq 0.05$).

662 **Table 5.** Caecal microbial counts at 42 days of age ¹

Treatment	<i>Campylobacter</i> spp. ¹	<i>E. coli</i> ¹
	log10 cfu/g	log10 cfu/g
T1	5.86	7.83
T2	5.03	7.24
T3	3.81	7.05
T4	3.74	6.64
SEM	0.296	0.191
P-values for contrast ²		
1 vs 2	0.005	0.002
1 vs 3	<0.001	<0.001
1 vs 4	<0.001	<0.001
Linear	<0.001	<0.001

663 ¹All means are average of 20 (2 x culture plates/treatment). *Campylobacter* spp. cultured on
664 CCDA medium, *E. coli* cultured on chromogenic agar; CFU = colony forming unit; SEM =
665 standard error of the mean;

666 T1= Control; T2 = 0.02 g/kg TYPLEX[®] Chelate; T3 = 0.05 g/kg TYPLEX[®] Chelate; T4 =
667 0.20 g/kg TYPLEX[®] Chelate

668 ²Significant level (P≤0.05).

669 **Table 6.** Effect of experimental diets on the concentrations of volatile fatty acid
670 (VFA; mg/kg) in the caecal content of broilers at 42 d of age.
671

Item	Acetic Acid	n-Butyric Acid	Propionic Acid	n-Valeric acid	Iso Valeric Acid	Iso Butyric Acid	Total VFA†
Treatment							
T ₁	4785	1222	1048	179	214	126	7573
T ₂	6009	2139	1232	228	212	135	9954
T ₃	5979	2032	1199	255	310	175 ^b	9567
T ₄	5497	1728	1155	237	256	144 ^{ab}	9018
SEM	387.9	188.9	95.2	30.3	37	18.09	622.4
P values for contrast ¹							
1 versus 2	0.003	<0.001	0.059	0.115	0.965	0.635	<0.001
1 versus 3	0.003	<0.001	0.118	0.015	0.012	0.009	0.002
1 versus 4	0.072	0.010	0.266	0.062	0.252	0.331	0.024
Linear	0.091	0.022	0.342	0.040	0.058	0.108	0.050

672 Means represents 2 birds per pen and 10 pens /treatment.

673 ¹Significance level ($P \leq 0.05$).

674 SEM = Standard error of differences of means.

675 †Total VFA = sum of all individual volatile fatty acid (VFA).